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[71] 专利权人 本元正阳基因技术股份有限公司
地址 100176 北京市北京经济技术开发区永昌中路6号

[72] 发明人 吴小兵 侯云德 伍志坚 曹晖
审查员 孙广秀

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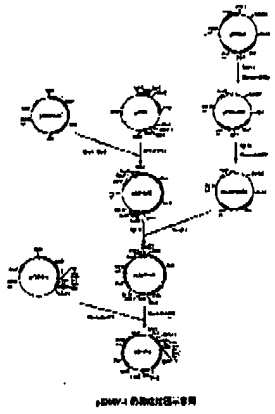
[54] 发明名称 系列通用型腺病毒伴随病毒载体的构建及用途

[57] 摘要

本发明涉及一系列通用型腺病毒伴随病毒(adeno-associated virus, AAV)载体的构建，这些载体包括pWAV-1、pWAV-2、pSNAV-1和pSNAV-2。其共同特征是每种载体均提供2型AAV两端的ITR、巨细胞病毒(CMV)立早增强子和启动子、多克隆位点和poly A信号。本发明提供了用上述通用型AAV载体构建携带外源基因的AAV载体质粒的方法。含有外源基因的AAV载体质粒既可用于产生重组AAV，又可直接作为真核表达质粒使用。pSNAV-1和pSNAV-2除具有上述共同特征外，还含有新霉素抗性基因表达盒。本发明利用这一特征，提供了用含有外源基因的pSNAV-1或pSNAV-2质粒建立稳定携带AAV载体的细胞株的方法。本发明进一步提供了"一株载体细胞/一株辅助病毒"的重组AAV生产方法，即用我们先前发明的一种用于重组AAV大规模生产的全功能辅助病毒(已申报专利，申请号98120033.8)感染AAV载体细胞株，实现重组AAV的大规模生产。



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Construction of a series of universal adeno-associated virus vectors and uses thereof

The present invention relates to the field of biotechnology.

Gene therapy, developed since 1990s, a brand new model for treating diseases, introduces therapeutic genes into human body to exert therapeutic effects. At present, more than 300 clinical solutions for gene therapy have been approved all over the world, and hundreds of thousands people have received such therapy. And the subjects for gene therapy have extended from the original genetic diseases and tumor into cardio-vascular diseases, infectious diseases, and so on.

The introduction of therapeutic genes into the cells of human body is the indispensable step, while suitable vectors are needed for the delivery of therapeutic genes. There are two kinds of vectors useful for the gene therapy, namely viral vectors and non-viral vectors. Among others, type 2 adeno-associated virus vector, a tiny virus defective in replication, is promising of being an ideal gene therapy vector due to its non-pathologic nature, abilities to infect cells post mitosis and integrate site-directedly into human chromosome 19, and so on, and thus are highly regarded in recent years in gene therapy researches. The conventional methods for preparing a recombinant adeno-associated virus (rAAV) carrying exogenous genes involve two plasmids: one is a vector plasmid carrying exogenous genes (i.e., therapeutic genes) expression cassette and AAV-2 inverted terminal repeats (ITR), in which ITR are the shortest cis-acting sequence; the other is a helper plasmid containing AAV-2 rep and cap genes, which provides trans-proteins necessary for the replication and package of rAAV. Co-transfection of these two plamids into the cell, followed by infection with helper virus (such as adenovirus or herpes simplex virus), would package a rAAV pseudovirus particle containing the exogenous genes. We have invented a full-function helper virus for large scale production of rAAV (CN Appl. No. 98120033.8), which simplified the production steps and increased the yields.

The first step for preparation of rAAV needs the construction of a AAV vector plasmid carrying the therapeutic genes. At present, a conventional method for construction of a AAV vector plasmid starts from a plasmid comprising the whole genome of AAV-2 (e.g., Sub201), unloading the rep and cap genes therein, maintaining only the ITRs on the both ends, then assembling sequentially the promotor, therapeutic genes, and poly A singal, which is to be improved for complexed steps and high cost.

The present invention simplifies the steps for constructing a AAV vector plamid carrying the therapeutic genes by providing a series of universal AAV vector plasmids and their construction method, as well as the methods for constructing a AAV vector plamid carrying the therapeutic genes by using said universal AAV vector plasmids.

The present invention relates to the construction of a series of universal AAV vector plasmids, which include pWAV-1, pWAV-2, pSNAV-1 and pSNAV-2. The common character thereof lies in that each vector provides ITRs from both ends of type 2 AAV, cytomegalovirus (CMV) immediate early enhancer and promoter, polyclonal sites, and polyA singal. The present invention provides a method to construct AAV vectors carrying exogenous genes using said universal AAV vectors. AAV vectors

carrying exogenous genes can be used not only for the production of recombinant AAVs, but also directly as eukaryotic expression plasmid. Besides, each of pSNAV-1 and pSNAV-2 additionally comprises a neomycin resistant gene cassette. Accordingly, the present invention provides a method for establishing a cell strain that carries stably the AAV vectors by using the exogenous gene-containing pSNAV-1 or pSNAV-2. The present invention further provides a method for production of recombinant AAVs via "one vector cell/one hepler virus", that is to say, infecting AAV vector cell strain using a full-function hepler virus invented earlier by us (CN Appl. No. 98120033.8) to realize a large-scale production of recombinant AAVs.

The original biological materials used in the invention are:

pSub201: a plasmid presented by Samulski laboratory containing the whole AAV-2 genome;

pUCMA, a plasmid made prviously by the present laboratorym, comprising CMV immediate early enhancer and promoter, polyclonal sites, and polyA;

pCMV/HyTK, an eukaryotic expression plasmid comprising CMV immediate early enhancer and promoter, and a HyTK gene under the control thereof;

pAV53, a AAV vector plamid carrying ampicillin resistant gene and E. coli replication origin;

pSV2neo, an eukaryotic expression plasmid manufactured by Promega expressing neomycin resistant gene.

pCMV-lacZ, an eukaryotic expression plasmid made prviously by the present laboratorym, comprising CMV immediate early enhancer and promoter, and a β-galactosidase gene under the control thereof;

pCD2, a retrovirus vector plasmid comprising CMV immediate early enhancer and promoter, and an E. coli cytosine deaminase (CD) gene under the control thereof, as well as a neomycin resistant gene under the control of the SV40 early promoter.

The host bacterium for the above plasmids is Escherichia coli MAX EFFICIENCY DH5α (GIBCO #18528-012)

The construction and characters of the series of universal AAV vector plasmids

The construction and characters of pWAV-1

See fig. 1. Rep and cap genes from pSub201 were cut by Xba I to leave the backbone of the plasmid and the ITRs on both ends of AAV-2 genome. And the CMV immediate early enhancer and promoter, polyclonal sites, and polyA sequences were unloaded from pUCMA by XbaI, then loaded between the above two ITRs, resulting the recombinant AAV vector plasmid pWAV-1.

This vector comprises ITRs from both ends of AAV-2, between the two ITRs sequentially are CMV immediate early enhancer and promoter, chimeric intron, polyclonal sites, and polyA singal. Wherein, the CMV immediate early enhancer and promoter, together with the chimeric intron are 1.1kb long. Said chimeric intron is consisted of the 5' splicer donor sites of the first intron from human β-globin, and the 3' splicer receptor sites of the variable region of heavy chain of immunoglobulin. Said chimeric

intron is placed upstream of the genes to be inserted, with the aim to splice mRNA precursor into mature mRNA at this site, as well as to prevent any possible splicing by a potential 5' splicer donor sites internal of the genes inserted. The polyclonal sites of said vector include such enzyme sites as Nhe I, Xho I, Pst I and BamH I, etc., which is convenient for the insertion of exogenous genes. The backbone of this plasmid is pEMBL, comprising the replication origin sequence necessary for replication in E. coli, as well as an ampicillin resistant gene. Since the maximal package limitation of rAAV is about 5kb, while said two ITRs, CMV immediate early enhancer and promoter, chimeric intron, polyclonal sites, and polyA singal are totally about 1.7kb, thus the length of exogenous genes that can be included is less than 3.3kb.

The construction and characters of pWAV-2

See fig. 2. HyTK gene was unloaded from pCMV/HyTK by double cut of Nhe I and Hind III, followed by recovery of the rest fragment of 2.9kb, with the sticky ends blunted by Klenow large fragment DNA polymerase and dNTP, then slef-ligated to constitute the recombinant pCMVPA. This pCMVPA was double cut by Xho I and BamH I to recover CMV and polyA fragment of 950bp. pAV53 was double cut by Xho I and BamH I to unload ampicillin resistant gene and E. coli replication origin while maintaining the backbone of the plasmid as well as the ITRs from both ends of AAV-2, which is ligated with the above CMV and polyA fragment to yield pWAV-2.

pWAV-2 also comprises ITRs from both ends of AAV-2, between the two ITRs sequentially are CMV immediate early enhancer and promoter, polyclonal sites, and polyA singal. The backbone of this plasmid contains the replication origin sequence necessary for replication in E. coli, as well as an ampicillin resistant gene. The polyclonal sites include Kpn I, EcoR I, Sal I, etc. The length of exogenous genes that can be included is less than 3.6kb.

The construction and characters of pSNAV-1

See fig. 3. Through a two-step cloning upon enzyme disgestion, blunting and re-ligation, the EcoR I and Bgl II sites were removed pSV2neo to yield pSV2neoΔEΔB. The LacZ expression cassette under the control of CMV promoter was cut from pCMV-lacZ by Xho I and BamH I, recovered, and then ligated into pAV53 between the ITRs resluted from double cut by Xho I and BamH I, forming the AAV vector plasmid pAV-LacZ carrying LacZ expression cassette. Said pAV-LacZ was cut by Bgl II to recover ITR-CMV-LacZ-ITR fragment, which was ligated at BamH I site into pSV2neoΔEΔB to form another AAV vector plasmid pSNAV-lacZ carrying LacZ expression cassette. Said pSNAV-lacZ was double digested by Xho I and BamH I to remove the LacZ expression cassette therein, which was replaced by CMV and polyA fragment obtained from pCMVPA upon double digestion of Xho I and BamH I, thereby resulted in the pSNAV-1.

pSNAV-1 comprises ITRs from both ends of AAV-2, between the two ITRs sequentially are CMV immediate early enhancer and promoter, polyclonal sites, and polyA singal. Besides, there is a neomycin resistant gene expression cassette under the control of SV40 promoter outside of the ITRs.

The backbone of this plasmid contains the replication origin sequence necessary for replication in E. coli, as well as an ampicillin resistant gene. The polyclonal sites include Kpn I, EcoR I, Sal I, Bgl II, etc. The length of exogenous genes that can be included is less than 3.6kb.

The construction and characters of pSNAV-2

See fig. 4. pCD2 was digested with Nhe I to unload CD-SV40-neo^r region, which was then inserted in forward direction into the Nhe I site of pWAV-1, thereby resulted in pWCDN. Said pWCDN was enzyme digested with BamH I to unload SV40-neo^r region, which was then assembled into the BamH I site of pWAV-2, thereby resulted in pSNAV-2.

Between the two ITRs on pSNAV-2, sequentially exists CMV immediate early enhancer and promoter, polyclonal sites, polyA singal, SV40 early promoter, a neomycin resistant gene expression cassette, and polyA singal. The backbone of this plasmid contains the replication origin sequence necessary for replication in E. coli, as well as an ampicillin resistant gene. The polyclonal sites include Kpn I, EcoR I, Sal I, etc. The length of exogenous genes that can be included is less than 1.9kb.

The above-mentioned four universal AAV vector plasmids are each preseved in Escherichia coli MAX EFFICIENCY DH5α (GIBCO #18528-012), which is subcultured at 37°C in LB medium containing 50-100 μg/ml ampicillin. The bacterium containing the plasmid pWAV-1 is named as DH5α/pWAV-1 (deposited under China General Mcirobiological Culture Collection Center with the accession number CGMCC No.0415.1). The bacterium containing the plasmid pWAV-2 is named as DH5α/pWAV-2 (deposited under China General Mcirobiological Culture Collection Center with the accession number CGMCC No.0415.2). The bacterium containing the plasmid pSNAV-1 is named as DH5α/pSNAV-1 (deposited under China General Mcirobiological Culture Collection Center with the accession number CGMCC No.0415.3). The bacterium containing the plasmid pSNAV-2 is named as DH5α/pSNAV-2 (deposited under China General Mcirobiological Culture Collection Center with the accession number CGMCC No.0415.4).

The usage of the series of universal AAV vector plasmids

For construction of recombinant AAV vector plasmids carrying exogenous genes

Depending on the size and the terminal enzyme restriction sites of the exogenous genes, selection can be made among pWAV-1, pWAV-2, pSNAV-1, or pSNAV-2 for loading. The specific procedure is to use restriction endonuclease corresponding to the polyclonal sites to cut the universal AAV vector plasmids, followed by ligation with exogenous genes treated by corresponding enzymes (e.g., T4 ligase), then using the resulted plasmid to transform competent E. coli. Upon screen of the recombinants, recombinant AAV vector plasmids carrying exogenous genes could be obtained.

For preparation of recombinant AAV carrying exogenous genes

Depending on the size and the terminal enzyme restriction sites of the exogenous genes, selection can be made among pWAV-1,

pWAV-2, pSNAV-1, or pSNAV-2 for loading. The pWAV-1, pWAV-2, pSNAV-1, or pSNAV-2 loaded with exogenous genes was co-transfected into cells together with a helper plasmid containing AAV-2 rep and cap genes using liposome (or calcium phosphate, or electroperforation), followed by infection with helper virus (such as adenovirus or herpes simplex virus), thereby a rAAV pseudovirus particle containing the exogenous genes could be packaged. Alternatively, using the full-function helper virus invented by us (CN Appl. No. 98120033.8) to infect cells having been transfected by pWAV-1, pWAV-2, pSNAV-1, or pSNAV-2 loaded with exogenous genes, a rAAV pseudovirus particle containing the exogenous genes could also be packaged.

pSNAV-1 or pSNAV-2 loaded with exogenous genes can be used to transfect cells using liposome or calcium phosphate, then screen cells on G418 pressure to give a G418 resistant cell, which is the recombinant AAV vector cell strain, this is because there is a neomycin resistant gene expression cassette in both pSNAV-1 and pSNAV-2. Using the full-function helper virus according to our invention to infect said cell strain, could package rAAV pseudovirus particles containing the exogenous genes.

For expression of exogenous genes in eukaryotic cells

Depending on the size and the terminal enzyme restriction sites of the exogenous genes, selection can be made among pWAV-1, pWAV-2, pSNAV-1, or pSNAV-2 for loading. Exogenous genes could be transiently expressed by transfecting mammal cells with pWAV-1, pWAV-2, pSNAV-1, or pSNAV-2 loaded with exogenous genes using liposome (or calcium phosphate, or electroperforation). pSNAV-1 or pSNAV-2 loaded with exogenous genes can be used to transfect mammal cells using liposome (or calcium phosphate, or electroperforation), then screen cells on G418 pressure to give a G418 resistant cell, which can enable the stable expression of exogenous genes.

EXAMPLES

The usages of the series of universal AAV vector plasmids are specified in the following examples, which should not be construed as limitations for the contents of the invention.

Example 1 Preparations of plasmid DNA

Plasmid DNA were extracted by the method of alkaline lysis according to Molecular Cloning –A Laboratory Manual, 2nd edition (Sambrook J. et al, 1986), and purified by the method of polyethylene glycol precipitation

Example 2 pWAV-1 is used to load E. coli cytosine deaminase (CD) gene

pCD2 was digested with Nhe I to unload CD-SV40-neo^r region of 2.8kb, separated by electrophoresis, then recovered and purified using glassmilk. pWAV-1 was digested with Nhe I for linearization, then it was ligated to CD-SV40-neo^r. Ligations were used to transform Escherichia coli MAX EFFICIENCY DH5α. The recombinants were identified by enzyme digestions, and a AAV vector plasmid pWCDN carrying E. coli cytosine

deaminase (CD) gene was screened.

Example 3 pSNAV-1 is used to load green fluorescence protein (GPF) gene

GFP was unloaded from pGreen Lantern-1 (purchased from GIBCO BRL) using Not I, and assembled into Not I site of pCDNA2.1 (purchased from INVITROGEH) to form recombinant plasmid pcDNA2.1/GFP(+/-).The recombinant plasmid which had the opposite orientation between GFP transcription and T7 promoter was named pcDNA2.0/GFP(-). The pcDNA2.0/GFP(-) was double digested by EcoR I and Xho I to recover the fragment GFP, which was inserted into the site between EcoR I and Sal I in pSNAV-1 to construct pSNAV–1-GFP carrying GFP. The plasmid comprised in turn the following elements: ITR-CMV-GFP-SV40 polyA-ITR-SV40 promoter-neo-polyA-amp^R-E.coli ori.

Example 4 pSNAV-2 is used to load green fluorescence protein (GPF) gene

The plasmid DNA PcDNA2.1A/GFP(-) was double cut by Kpn I and Xho I to recover the fragment GFP, which was inserted into the site between Kpn I and Sal I in pSNAV-2 to construct pSNAV–2/GFP. The plasmid comprised in turn the following elements: ITR-CMV-GFP-SV40 polyA-SV40 promoter-neo-polyA-ITR-amp^R-E.coli ori.

Example 5 the establishment of recombinant AAV vector cell strian carrying green fluorescence protein (GPF) gene

1.5 μg pSNAV–1-GFP and pSNAV–2-GFP were used individually to transfect 50% confluenced BHK-21 cells cultured in 6-well plate using 10 μl Lipofactamine (GIBCO BRL) according to the product description. Medium is changed after 24hrs, and cells were grown in 1640 medium (supplemented with 10% fetal bovine serum) containing 400 μg/ml G418. The resulting resistant cells after about 10 to 15 days are recombinant AAV vector cell strains carrying green fluorescence protein (GPF) gene.

Example 6 the production of recombinant AAV-GFP by cotransfection of plasmids

1.5 μg pSNAV–1-GFP (or pSNAV–2-GFP) was mixed with 3μg pAAV/Ad to transfect 80% confluenced BHK-21 cells cultured in 6-cm plate using 20 μl Lipofactamine (GIBCO BRL) according to the product description. Medium is changed every 5hrs, and cells were infected by type 5 adenovirus (MOI=2). Upon complete pathologic at 48-72hrs, the cells were disrupted for four times by freezing and thawing cycles, and cell debris were removed by low speed centrifugation, the supernatant was collected and subjected to heat at 56□ to inactivate the helper virus, then stored at -20□ until use.

Example 7 the production of recombinant AAV-GFP by infecting AAV-GFP vector cell strain using full-function helper virus

Full-function helper viruses at MOI of 0.1 were used to infect recombinant AAV vector cell strains carrying green fluorescence protein (GPF) gene established in Example 5. Upon complete pathologic at 24-48hrs, the cells and medium were subjected to four cycles of freezing and thawing, centrifuged at 100rpm/min for 5 min, the resulting

supernatant contained lots of rAAV-GFP viruses. Thus infectious rAAV-GFP virus could be conveniently produced by such a process, and batch production of rAAV could be realized.

Example 8 the transduction of cultured cells with recombinant AAV-GFP

1 ml rAAV-GFP virus supernatant was removed, and added into cultured BHK cells (80% confluence), lots of green cells could be observed under fluorescent microscopy (light excited is of 490 nm) after 24-48 hrs. It showed that rAAV thus produced is infectious, and capable of introducing exogenous genes into cells.

Example 9 the transient expression of GFP in cells

1.5 μg pSNAV–1-GFP (or pSNAV–2-GFP) was used to transfect 80% confluenced BHK-21 cells cultured in 6-cm plate using 20 μl Lipofactamine (GIBCO BRL), lots of green cells could be observed under fluorescent microscopy (light excited is of 490 nm) at 24 hrs. It showed that pSNAV–1-GFP (or pSNAV–2-GFP) can express GFP in eukaryotic cells, and that both pSNAV–1 and pSNAV–2 could be used as eukaryotic expression vectors.

Example10 the stable expression of GFP in cells

1.5 μg pSNAV–1-GFP and pSNAV–2-GFP were used individually to transfect 50% confluenced BHK-21 cells cultured in 6-well plate using 10 μl Lipofactamine (GIBCO BRL) according to the product description. Medium is changed after 24hrs, and cells were grown in 1640 medium (supplemented with 10% fetal bovine serum) containing 400 μg/ml G418. The resulting resistant cells after about 10 to 15 days are found to be green in color under fluorescent microscopy (light excited is of 490 nm), that is to say, GFP was expressed. GFP was continuously expressed with the passage of cells, which showed that both pSNAV–1 and pSNAV–2 could be used as eukaryotic expression vectors to mediate stable expressions of exogenous genes.

Figure 1 construction illustration of pWAV-1;
Figure 2 construction illustration of pWAV-2;
Figure 3 construction illustration of pSNAV-1;
Figure 4 construction illustration of pSNAV-2.

What we claimed is:

The present invention relates to the field of biotechnology, particularly to a series of universal adeno-associated virus (AAV) vector plasmids useful for gene transfer, gene therapy, and eukaryotic gene expressions.

1. Universal AAV vector plasmids pWAV-1 and pWAV-2 according to the present invention, characterized by comprising ITRs from both ends of type 2 AAV, between which ITRs sequentially are cytomegalovirus immediate early enhancer and promoter, polyclonal sites, and polyA singal.

2. Universal AAV vector plasmid pSNAV-1 according to the present invention, characterized by comprising ITRs from both ends of type 2 AAV, between which ITRs sequentially are cytomegalovirus immediate early enhancer and promoter, polyclonal sites, and polyA singal, as well as a neomycin resistant gene cassette outside the ITRs.

3. Universal AAV vector plasmid pSNAV-2 according to the present invention, characterized by comprising ITRs from both ends of type 2 AAV, between which ITRs sequentially are cytomegalovirus immediate early enhancer and promoter, polyclonal sites, polyA singal, and a neomycin resistant gene cassette.

4. Universal AAV vector plasmids according to the present invention used to carry exogenous genes, characterized by insertion of said exogenous genes into the polyclonal sites of said universal AAV vector plasmids.

5. Universal AAV vector plasmids according to the present invention used to produce recombinant AAVs carrying exogenous genes.

6. Universal AAV vector plasmids according to the present invention used to establish cell strains stably carrying recombinant AAVs, said cell strains are characterized by comprising, stably integrated into their chromosomes, AAV ITRs and exogenous gene expression unit.

7. Universal AAV vector plasmids according to the present invention used for eukaryotic cells for exogenous gene expression.

8. Universal AAV vector plasmids according to claim 1, 2 or 3, wherein a SV40 early enhancer and promoter locates in front of the polyclonal sites.

9. Universal AAV vector plasmids according to claim 1, 2 or 3, wherein the long terminal repeat region from genome of Rous sarcomas virus locates in front of the polyclonal sites.

10. Universal AAV vector plasmids according to claim 1, 2 or 3, wherein the thydimine kinase promoter from herpes simplex virus locates in front of the polyclonal sites.

11. Universal AAV vector plasmids according to claim 2 or 3, wherein the selective marker gene is hygromycin resistant gene.

12. Universal AAV vector plasmids according to claim 2 or 3, wherein the selective marker gene is dihydrofolate reductase gene.

13. Universal AAV vector plasmids according to claim 2 or 3, wherein the selective marker gene is xanthine-guanine phosphoribosyl transferase gene.

14. Universal AAV vector plasmids according to claim 2 or 3, wherein the selective marker gene is adenosine deaminase gene.

ABSTRACTS

The present invention relates to the construction of a series of universal adeno-associated virus (AAV) vectors, which include pWAV-1, pWAV-2, pSNAV-1 and pSNAV-2. The common character thereof lies in that each vector provides ITRs from both ends of type 2 AAV, cytomegalovirus (CMV) immediate early enhancer and promoter, polyclonal sites, and polyA singal. The present invention provides a method to construct AAV vectors carrying exogenous genes using said universal AAV vectors. AAV vectors carrying exogenous genes can be used not only for the production of recombinant AAVs, but also directly as eukaryotic expression plasmid. Besides, each of pSNAV-1 and pSNAV-2 additionally comprises a neomycin resistant gene cassette. Accordingly, the present invention provides a method for establishing a cell strain that carries stably the AAV vectors by using the exogenous gene-containing pSNAV-1 or pSNAV-2. The present invention further provides a method for production of recombinant AAVs via "one vector cell/one hepler virus", that is to say, infecting AAV vector cell strain using a full-function hepler virus invented earlier by us (CN Appl. No. 98120033.8) to realize a large-scale production of recombinant AAVs.